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Identification and Characterization of Two Subpopulations of *Encephalitozoon intestinalis*

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Microsporidia are obligate intracellular protozoa that have been shown to be pathogenic to most living creatures. The development of in vitro cell culture propagation methods has provided researchers with large numbers of spores and facilitated the study of these organisms. Here, we describe heterogeneity within cell culture-propagated *Encephalitozoon intestinalis* suspensions. Flow cytometer histograms depicting the log side scatter and forward-angle light scatter of spores from nine suspensions produced over 12 months consistently showed two populations differing in size. The suspensions were composed primarily of the smaller-spore subpopulation (76.4% ± 5.1%). The presence of two subpopulations was confirmed by microscopic examination and image analysis ($P < 0.001$). Small subpopulation spores were noninfectious in rabbit kidney (RK13) cell culture infectivity assays, while the large spores were infectious when inocula included ≥25 spores. The small spores stained brilliantly with fluorescein isothiocyanate-conjugated monoclonal antibody against *Encephalitozoon* genus spore wall antigen, while the large spores stained poorly. There was no difference in staining intensities using commercial (MicroSporFA) and experimental polyclonal antibodies. Vital-dye (DAPI [4′,6′-diamidino-2-phenylindole], propidium iodide, or SYTOX Green) staining showed the spores of the small subpopulation to be permeable to all vital dyes tested, while spores of the large subpopulation were not permeable in the absence of ethanol pretreatment. PCR using primers directed to the 16S rRNA or β-tubulin genes and subsequent sequence analysis confirmed both subpopulations as *E. intestinalis*. Our data suggest that existing cell culture propagation methods produce two types of spores differing in infectivity, and the presence of these noninfective spores in purified spore suspensions should be considered when designing disinfection and drug treatment studies.

Microsporidia are a diverse group of spore-forming protozoa which includes over 1,200 species belonging to 143 genera. These organisms have been shown to be pathogens of a plethora of vertebrate and invertebrate hosts. At least 13 microsporidia, including members of the genera *Entercytozoon* and *Encephalitozoon*, have been shown to cause disease in humans, particularly among those with compromised immune systems (3, 15, 24). *Enterocytozoon bienusi*, *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi*, and *Encephalitozoon hellem* are the most common microsporidian human pathogens and are associated with gastroenteritis, keraconjunctivitis, respiratory tract infections, hepatitis, peritonitis, sinusitis, nephritis, prostatitis, and encephalitis (9). The infectious form of these organisms is a small (~2-μm-diameter), environmentally resilient spore that has demonstrated resistance to removal by conventional water treatment practices (11). Recognizing the potential of microsporidia to be transmitted through drinking water, the Environmental Protection Agency placed microsporidian species on the Candidate Contaminant List 1 for drinking water (7).

In vitro culturing of *Encephalitozoon* sp. spores, with purification by Percoll gradient separation, has provided researchers with large numbers of highly purified spores (22). Spores purified by these methods have been used to test disinfectant (13, 14, 19, 25) and therapeutic-agent efficacies (6), to define the immunologic responses of microsporidium-infected animals (6a), to determine parasite physiological and biochemical responses (16), to test primers for use in molecular studies and detection methods (1, 5), and to develop antibodies (2, 18). However, little is known about the comparability of these in vitro-cultured organisms to those occurring naturally. Thellier et al. (21) reported that antibodies directed against cell culture-propagated *E. intestinalis* did not stain *E. intestinalis* spores isolated from fecal specimens, indicating that there are differences between naturally produced and laboratory-propagated spores. Hayman et al. (12) also identified variability within purified spore suspensions and characterized differences in surface wall protein expression of spore types.

Here, we describe variability within *E. intestinalis* suspensions produced using cell culture and Percoll gradient separation and demonstrate differences in morphology, antibody labeling, vital-dye permeability, and cell culture infectivity within these subpopulations.

MATERIALS AND METHODS

Spore production. Spores of *E. cuniculi* ATCC 50502, *E. hellem* ATCC 50451, and a duodenal isolate of *E. intestinalis*, ATCC 50603, were purified weekly from constantly infected RK13 cell monolayers (25). After enumeration by hemacytometer, the spore concentration was adjusted to 10^7/ml/tube, and the spores were stored in phosphate-buffered saline (PBS) or reagent-grade water at 4°C. Additional *E. intestinalis* and *E. cuniculi* isolates propagated and purified using

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previously described methods (7) were obtained from Tulane University, New Orleans, La. (courtesy of E. Didier) for comparative evaluation.

Antibody labeling. Monoclonal antibody (MAb 7G7) reactive to surface wall protein 2 (SWP2) (12) was produced in mice (18) and provided by the National Institutes of Health (courtesy of T. Nash). The antibody was conjugated to fluorescein isothiocyanate (FITC) by a commercial source (Cell Essentials, Boston, Mass.). FITC-conjugated polyclonal antiserum to *E. intestinalis* spores (MicroSporFA) was purchased from Waterborne Inc. (New Orleans, La.). Antibody labeling was performed by combining 10^5 *E. intestinalis* spores with either a 1:100 dilution of MAb 7G7 or a 1:20 dilution of MicroSporFA in 12- by 75-mm cell culture tubes. The suspensions were vortexed, incubated for 30 min at room temperature, and analyzed by flow cytometry. Indirect antibody labeling methods were performed using an affinity-purified polyclonal rabbit anti-*E. cuniculi*, -*E. hellem*, and -*E. intestinalis* antibody generated against UV-treated spores. The purified spores (10^5) were incubated for 45 min at 37°C with polyclonal antibody at a final concentration of 1:50. After being washed with 1 ml of 18-MΩ water (14,100 × *g*; 5 min), the spores were suspended in 100 μl of Cy3-conjugated goat anti-rabbit immunoglobulin G (KPL, Gaithersburg, Md.) that had been diluted 1:10 in PBS supplemented with 0.01% Tween 80 and 1% goat serum. The suspensions were incubated for 45 min at 37°C before analysis by flow cytometry.

FCCS. Flow cytometric analysis and cell sorting (FCCS) was performed on an EPICS Elite flow cytometer (Beckman Coulter, Fullerton, Calif.) equipped with a 488-nm-wavelength argon ion laser. Forward scatter was detected on a linear scale, whereas side scatter and fluorescence detectors were set to a logarithmic scale. Green-fluorescence (FITC) emissions were detected using a 525-nm-wavelength bandpass filter. Data from a minimum of 5,000 events were collected.

Precisely enumerated spore suspensions were prepared by FCCS methods routinely used in our laboratory for the preparation of *Cryptosporidium* and *Giardia* standards (R. M. Hoffman, unpublished data). Briefly, spores were identified using nonfluorescent light scatter measurements (log side and forward-angle light scatter), and the instrument was programmed to sort the target number of spores either into tubes containing 18-MΩ water or onto three-well glass microscope slides. The accuracy and precision of the cytometer were determined by sorting spores onto three-well microscope slides and enumerating them microscopically (model E400; Nikon USA, Melville, N.Y.). Purified *E. intestinalis* subpopulations were used in the methods described below.

Vital-dye staining. (i) DAPI. A stock solution of 5 μg of DAPI (4′,6 diamidino-2-phenylindole) (Sigma, St. Louis, Mo.)/ml was prepared in absolute methanol and stored at 4°C shielded from light. The stock solution was diluted 1:5 with reagent grade water, and 40 μl was added to suspensions containing 10^5 spores that had been prelabeled with MAb as described previously. The spores were flow sorted onto Teflon-coated three-well microscope slides (Erie Scientific, Portsmouth, N.H.), and the DAPI fluorescence was examined by fluorescence microscopy. Spores were also stained using a modified DAPI staining protocol in which ~10^5 spores were concentrated by centrifugation (12,000 × *g*; 2 min) and resuspended in a 1:1 mixture of 95% ethanol and buffer containing 0.01 M Trisma, 0.01 M EDTA, and 0.1 M NaCl (pH 7.2). After being incubated at 70°C for 30 min, the spores were centrifuged, the supernatant was decanted, and the pellet was resuspended in 200 μl of 18-MΩ water containing 0.01% Tween 20, 10 μl of MicroSporFA, and 40 μl of DAPI. The spores were incubated for 45 min at room temperature in the dark before cell sorting and microscopic examination as described above.

(ii) PI. A propidium iodide (PI) solution (500 μg/ml; Sigma) was prepared in PBS (pH 7.2), sterile filtered, and stored in the dark at 4°C. One hundred microliters of the stock solution that had been diluted 1:10 in PBS supplemented with 0.01% Tween 80 and 1% goat serum was added to 10^5 spores prelabeled with MAb as described above. The spores were analyzed by flow cytometry and fluorescence microscopy after a 15-min room temperature incubation.

(iii) SYTOX Green. SYTOX Green (Molecular Probes, Eugene, Ore.) staining methods were adapted from previously published methods (10). Briefly, spores were labeled with rabbit polyclonal antibody and Cy3-conjugated goat anti-rabbit immunoglobulin G as described previously and washed to remove unbound secondary antibody. The spore pellet was resuspended in 100 μl of a 50 μM SYTOX Green solution, incubated for 30 min at room temperature, and washed twice with 1 ml of reagent-grade water prior to flow cytometric analysis and cell sorting. Heat-inactivated spores (70°C; 30 min) were used as positive staining controls in all vital-dye staining experiments.

Spore measurement. Slides containing flow cytometer-sorted *E. intestinalis* spores were mounted with DABCO-glycerol mounting medium and examined under light microscopy at ×1,000 using a Nikon E400 microscope. Digital photomicrographs were captured using a charge-coupled device camera (Magnafire; Optronics, Goletta, Calif.) and analyzed with image analysis software (ImagePro-Express; Media Cybernetics, Silver Springs, Md.). The widths and lengths of 20 spores per subpopulation were measured. The results were imported into Microsoft Excel spreadsheets, where mean differences were analyzed for statistical significance according to Student's *t* test.

Cell culture infectivity methods. Ten-milliliter suspensions of *E. intestinalis* spores ranging from 10 to 1,000/ml of reagent grade water were prepared using FCCS and shipped to the University of Arizona for infectivity characterization. One milliliter of each spore dilution was inoculated onto 3-day-old RK13 cell monolayers that were grown on 15-mm-diameter sterile Thermanox coverslips in 24-well plates according to the method described by Wolk et al. (25). Either 5 or 10 coverslips were inoculated for each spore dilution, with five uninoculated coverslips serving as negative controls. Following inoculation, the 24-well plates were centrifuged at 2,300 × *g* for 10 min and incubated for 6 days at 35°C in a humidified 5% CO_2 atmosphere. The coverslips were removed from the wells, fixed with methanol, stained with Giemsa stain, and mounted with Gel/Mount (biomeda, Foster City, Calif.) on glass slides. Evidence of infection was determined by scanning the entire surface area of each coverslip at ×400 magnification by light microscopy for the presence of parasitophorous vacuoles containing mature microsporidian spores in the cytoplasm. Coverslips showing one or more infected cells were scored as positive. The percent infection was calculated by dividing the number of positive (infected) wells by the number of wells inoculated.

PCR. Two hundred microliters of a suspension containing FCCS-purified spores from the small- or large-spore subpopulation was placed in a 1.5-ml microcentrifuge tube and microwaved for 30 s at high power to lyse the spores. A QIAamp (Qiagen Inc., Valencia, Calif.) kit was used to isolate DNA from the lysed spores. The DNA was concentrated using a YM100 Microcon filter (Millipore, Bedford, Mass.) and stored at 4°C. *E. intestinalis* subpopulations were evaluated using the 16S rRNA primers SI500 (23) and PMP1/VI (8) and β-tubulin primers developed for this work (forward, 5′-GGCTGCACTTCTTCGTTGTTGG-3′, and reverse, 5′-CGGTGCAATGTCACAAACTGC-3′). PCRs were performed in a final volume of 50 μl containing 0.2 μM concentrations of either the SI500-PMP1/VI or β-tubulin primer set; 1× PCR Gold polymerase buffer; 2.5 mM $MgCl_2$; 200 μM (each) dATP, dCTP, and dGTP and 400 μM dUTP; 1.25 U of AmpliTaq Gold DNA polymerase; 0.2 U of AmpErase (Applied Biosystems Inc., Foster City, Calif.); ~10 to 100 ng of template DNA; and sterile double-distilled water. The samples were amplified using a Mastercycler gradient thermal cycler (Eppendorf Scientific, Inc., Westbury, N.Y.). Samples were initially incubated at 52°C for 2 min to allow the AmpErase to destroy any contaminating amplicon containing dUTP. An initial denaturation step was performed at 95°C for 10 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and elongation at 72°C for 30 s. The samples were subjected to a final 10-min elongation step at 72°C. The PCR products were electrophoresed in 1.2% agarose gels, stained with 0.5 g of ethidium bromide/liter, and photographed under UV light.

Two negative control tubes containing 10 μl of sterile double-distilled water instead of DNA were the first and last samples completed with each PCR round. A control tube containing 10^3 purified *E. intestinalis* spores in 10 μl of sterile double-distilled water was used as a positive template control.

The 375-bp 16S rRNA and the 288-bp β-tubulin amplicons were purified using the QIAquick PCR purification kit (Qiagen Inc.) and sequenced by automated DNA sequencing with the forward and reverse PCR primers. Sequencing reactions were performed by the DNA Sequencing Facility at The University of Arizona, using a model 377 DNA sequencer (Applied Biosystems Inc.).

RESULTS

Figure 1 depicts two-parameter plots of the log side scatter and forward-angle light scatter of freshly harvested and purified *E. intestinalis* spores. This profile displays two clearly resolved subpopulations within the Percoll-purified spore suspension. Data from nine lots purified over a 12-month period showed that spores in these suspensions predominantly had a smaller, more ovoid morphology (76.4% ± 5.1%), while the larger, more elongated spore subpopulation comprised only 16.4% ± 3.0%. The above observation was confirmed using spores produced by researchers at Tulane University, although the prevalence of large spores in the Tulane preparation was slightly greater than that in the University of Arizona spores (data not shown).

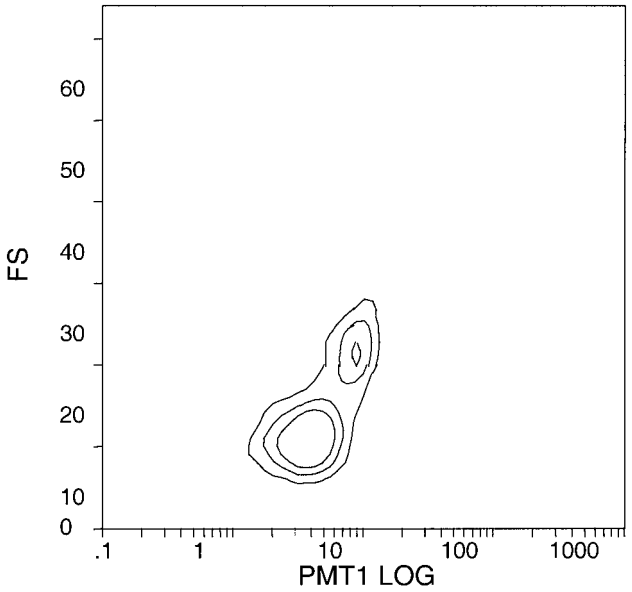

FIG. 1. Flow cytometer-generated contour plot depicting log 90° light scatter versus forward-angle light scatter (FS) of cell culture-propagated, Percoll-purified *E. intestinalis* spores.

Spore measurement. Length measurements of the small- and large-spore subpopulations were statistically different ($P < 0.001$). The average length of the small spores was 2.38 ± 0.03 μm, while the large spores measured 2.69 ± 0.04 μm ($n = 20$). There was no statistical difference between the widths of the two spore subpopulations (data not shown).

Antibody staining. There were significant differences between the fluorescence intensities of the *E. intestinalis* spore subpopulations when stained with FITC-conjugated MAb 7G7 ($P < 0.001$) and purified by flow cytometry using forward-angle light scatter measurements (Fig. 2). Small spores stained brilliantly (4+) with this MAb, while large spores stained weakly (1+). There was no difference in fluorescence intensities when the spores were stained with MicroSporFA or if the spores were stained with the experimental polyclonal and Cy3-conjugated secondary antibodies (data not shown).

TABLE 1. RK13 cell culture infectivities of the large- and small-spore subpopulations

No. of spores/ inoculum	No. of coverslips infected with[a]:	
	Large spores	Small spores
500	5/5	0/5
100	4/5	0/5
50	5/10	0/5
25	1/10	ND[b]

[a] Number infected/total.
[b] ND, not determined.

Vital-dye staining. DAPI staining confirmed the presence of nuclear material in both spore subpopulations. Spores of the small-spore subpopulation readily took up the DAPI, while those of the large-pore subpopulation did not, unless ethanol pretreatment was incorporated into the staining protocol. Only the small-spore subpopulation was permeable to PI and SYTOX Green (data not shown).

Cell culture infectivity. Either 5 or 10 coverslips were infected with doses of 25 to 500 spores of each subpopulation, and the infectivity was scored. Spores of the large-spore subpopulation were infectious in cell culture infectivity assays with a dose as low as 25 spores (Table 1). However, repeated infections with spores isolated from the small-spore subpopulation showed that these spores were not infective, even at doses as high as 500 spores. The uninoculated coverslips showed no signs of infection (data not shown).

PCR. PCR was performed on spores from the large- and small-spore populations using primers directed against the 16S

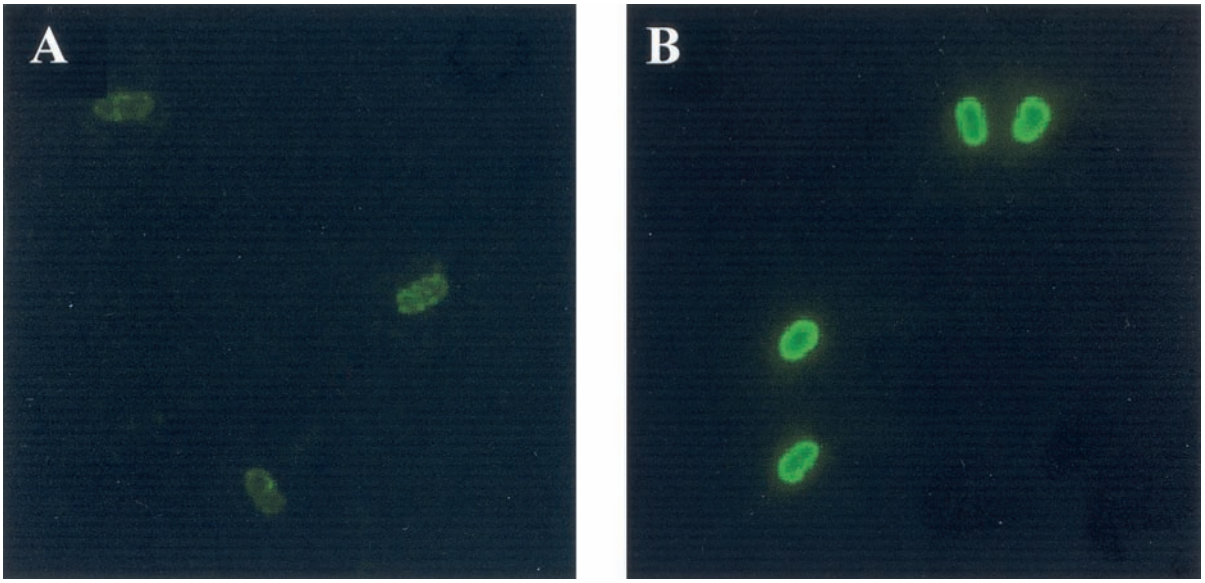

FIG. 2. *E. intestinalis* spores labeled with FITC-conjugated MAb 7G7. Subpopulations were isolated using flow cytometry with cell sorting. (A) Larger spores. (B) Smaller spores.

TABLE 2. Summary of differences between *E. intestinalis* subpopulations

Parameter	Values[d]	
	Large	Small
Size (μm)[a]	2.69	2.38
% of total population[b]	76.4	16.4
Vital-dye permeability		
DAPI	+[e]	+
PI	−	+
SYTOX Green	−	+
Infectivity (cell culture)	+	−
Antibody staining		
Bethyl PAb[c]	+++	+++
MicroSporFA	+++	+++
7G7	+	++++
Sequence analysis		
β-Tubulin	++	++
16S rRNA	++	++

[a] Statistically significant difference ($P < 0.001$).
[b] $n = 9$.
[c] PAb, polyclonal antibody.
[d] −, not reactive; +, weakly reactive; ++, reactive; +++, strongly reactive; ++++, very strongly reactive.
[e] Only with ethanol pretreatment.

rRNA and β-tubulin genes. The nucleotide sequences of the PCR products were determined and compared to those of the *E. intestinalis* 16S rRNA and β-tubulin genes registered in GenBank. The nucleotide sequences of the 16S rRNA genes amplified from the small- and large-spore populations were almost identical to one another, with the exception that the small-spore subpopulation 16S rRNA gene contained a single-base-pair substitution (A versus T) at position 28 of the amplicon relative to the large-spore subpopulation 16S rRNA gene. When the small- and large-spore population sequences were compared to those in the GenBank database, the top two matches were to *E. intestinalis* (GenBank accession numbers U09929 and L39113). The nucleotide sequences of the β-tubulin amplicons from both the small- and large-spore populations were identical to that of the *E. intestinalis* β-tubulin sequence in GenBank (accession number AF297876). These data indicate that the two spore populations were not derived from contaminants and were in fact spores of *E. intestinalis.*

CONCLUSIONS

In this study, we describe the presence of two subpopulations varying in size, infectivity, and vital-dye permeability within suspensions produced using standard propagation methods (Table 2). The existence of two spore subpopulations within Percoll-purified spore suspensions has been previously reported. Hayman et al. (12) identified two surface wall proteins (SWP1 and SWP2) localized in the exospore region of *E. intestinalis* and concluded that as the spores mature from meront to sporont, SWP2 expression (identified by MAb 7G7 labeling) became more prevalent on the spore surface while SWP1 was blocked or internalized. Morphologically, they identified the 7G7-positive (SWP2) spores as more fully formed ovoid spores compared to the more elongated SWP2-negative spores. In this study, we also report two spore populations demonstrating marked differences in MAb 7G7 staining. However, while both studies determined that the smaller, more ovoid spores reacted with this MAb, the present study shows that this subpopulation was not infectious in RK13 cell culture assays while the larger and slightly elongated spores, which stained only faintly with MAb 7G7, did cause infection in cell cultures, in one case with a dose as low as 25 spores. This infection disparity was confirmed with spores flow sorted in an independent flow cytometry facility.

Vital dyes, such as PI and SYTOX Green, have been used to assess the viabilities of a number of organisms, including parasitic protozoa (4, 17). While exclusion of these dyes cannot confirm viability, the penetration of the dyes into the organisms can only occur if the membrane has been compromised. In these studies, the small, ovoid, 7G7-positive spores were permeable to PI, SYTOX Green, and DAPI. Spores of the large-spore subpopulation did not stain with PI, SYTOX Green, or DAPI unless ethanol pretreatment was performed. Spores of closely related microsporida (*E. hellem* and *E. cuniculi*) also exhibited two subpopulations, the smaller of which stained PI and SYTOX Green positive (data not shown).

Flow cytometer histograms depicting log 90° light scatter versus linear forward-angle light scatter indicated that purified spore suspensions contained spores of two different sizes. Flow cytometric evaluation of purified *E. intestinalis* was previously performed (20). However, in these studies, the subpopulations were indiscernible, since forward-angle light scatter was plotted on a log scale. In this study, the subpopulations were sorted using FCCS, and the lengths and widths of each spore subpopulation was determined using image analysis software. The average length of spores sorted from the large-spore subpopulation was ~0.3 μm longer than that of spores isolated from the small-spore subpopulation ($P < 0.001$). This two-subpopulation profile was consistently observed in nine lots of *E. intestinalis* produced over a 1-year period, with the average number of noninfective, vital-dye-permeable spores in the preparation more than quadruple the number of infectious spores (76.4 versus 16.4%). The presence of two subpopulations was also observed using suspensions propagated and purified in an unrelated laboratory.

The molecular identity of the spores from the large- and small-spore subpopulations was confirmed using FCCS-purified spore suspensions. PCR analysis with subsequent sequence analysis indicated that both the large- and small-spore populations had 16S rRNA and β-tubulin genes that were identical to that of *E. intestinalis*, indicating that the two spore populations were not derived from contaminants.

Spore propagation and purification using commonly referenced methods (1, 2, 5, 6, 6a, 13, 14, 16, 18, 19, 25) yielded two subpopulations of *E. intestinalis*. The smaller, more prevalent spore subpopulation identified in these studies was not capable of initiating infection in cell culture infectivity assays and was shown to be permeable to vital dyes normally excluded by intact organisms. The morphological identity of this spore form has not been confirmed. It is possible that the smaller, more prevalent spore type is an artifact of tissue culture propagation methods, or perhaps Percoll purification methods copurify a meront-sporont transitional form. Electron microscopy studies performed using flow cytometrically isolated spore subpopulations may be helpful in positively identifying this spore subpopulation. This finding has significant implica-

tions for previous and future disinfection and drug efficacy studies, since these smaller, noninfective spores have been shown in the current studies to comprise the majority of the purified spore suspension. Failure to account for the large number of noninfectious spores in any given dose could result in a significant overestimation of viability reduction of 1 log unit or greater.

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